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Original Article

p53-Armed Oncolytic Virotherapy Improves Radiosensitivity in Soft-Tissue Sarcoma by Suppressing BCL-xL Expression

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Soft-tissue sarcoma (STS) is a heterogeneous group of rare tumors originating predominantly from the embryonic mesoderm. Despite the development of combined modalities including radiotherapy, STSs are often refractory to antitumor modalities, and novel strategies that improve the prognosis of STS patients are needed. We previously demonstrated the therapeutic potential of two telomerase-specific replication-competent oncolytic adenoviruses, OBP-301 and tumor suppressor p53-armed OBP-702, in human STS cells. Here, we demonstrate *in vitro* and *in vivo* antitumor effects of OBP-702 in combination with ionizing radiation against human STS cells (HT1080, NMS-2, SYO-1). OBP-702 synergistically promoted the antitumor effect of ionizing radiation in the STS cells by suppressing the expression of B-cell lymphoma-X large (BCL-xL) and enhancing ionizing radiation-induced apoptosis. The *in vivo* experiments demonstrated that this combination therapy significantly suppressed STS tumors' growth. Our results suggest that OBP-702 is a promising antitumor reagent for promoting the radiosensitivity of STS tumors.

Key words: soft-tissue sarcoma, radiotherapy, oncolytic adenovirus, p53, BCL-xL

Soft-tissue sarcoma (STS) is a heterogeneous group of rare tumors arising predominantly from the embryonic mesoderm [1]. There are over 100 different histological and molecular subtypes of STS [2]. Treatment for STS requires a multidisciplinary approach that involves surgery, chemotherapy, and radiotherapy, and these modalities are planned and delivered by a multidisciplinary sarcoma team [2]. Despite the development of combined modalities, poor response to radiotherapy is a predictive factor of local and systemic STS recurrence, which leads to poor prognoses [3]. Among the different histological subtypes of STS, Ewing sarcoma is relatively sensitive to radiotherapy, whereas other STS subtypes such as fibrosarcoma and malignant peripheral nerve sheath tumor are relatively less sensitive to radiotherapy. However, the mechanisms that underlie STSs sensitivity to radiotherapy are not fully understood.

As a standard antitumor modality, radiotherapy is used to induce significant apoptosis of STS cells [2], and

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STS patients' resistance to radiotherapy is a major obstacle to improving their clinical outcomes [3]. The B-cell lymphoma 2 (BCL2) family of proteins, including BCL2, myeloid cell leukemia 1 (MCL1), and B-cell lymphoma-X large (BCL-xL) plays a crucial role in the avoidance of radiotherapy-induced apoptosis in solid tumors [4,5]. Several clinical studies have shown that the inhibition of pro-survival BCL2 family proteins is a promising antitumor strategy for treating patients with solid tumors [6], indicating that BCL2-family proteins are attractive targets [7]. However, BCL2-family proteins are also required for the survival of normal cells, including hematopoietic stem cells [7]. It is therefore necessary to suppress the expression of pro-survival BCL2-family proteins only in malignant tumor cells without harming normal cells.

Oncolytic virotherapy is a novel antitumor modality used to induce significant lytic cell death without affecting surrounding normal cells [8]. One of our research group's earlier investigations demonstrated the therapeutic potential of two telomerase-specific replication-competent oncolytic adenoviruses, *i.e.*, OBP-301 and tumor suppressor p53-armed OBP-301 variant (OBP-702), for inducing cytopathic activity in a variety of human sarcoma cells, including osteosarcoma (OS) cells and STS cells [9,10]. We observed that p53-armed OBP-702 exerts more profound antitumor effects in association with autophagy and apoptosis in human OS cells compared to non-armed OBP-301 or a p53-expressing replication-deficient adenovirus (Ad-p53) [10]. In combination with chemotherapy, OBP-301 promoted the antitumor efficacy of various chemotherapeutic agents including cisplatin, doxorubicin, and zoledronic acid in human OS cells by suppressing the expression of MCL1 [11,12]. We also recently demonstrated that OBP-702 has the therapeutic potential to increase the chemosensitivity of human OS cells via a suppression of the expression of multidrug resistance protein 1 (MDR1) [13]. Since Xiao *et al.* demonstrated that Ad-p53 treatment efficiently improved the therapeutic efficacy of radiotherapy in patients with advanced STS [14], we hypothesized that OBP-702 would exhibit therapeutic potential to improve the radiosensitivity of human STS cells.

We thus conducted the present study to investigate the ability of OBP-702 to promote the radiosensitivity of human STS cells. The cytopathic activity of combination therapy, *i.e.*, OBP-702 and ionizing radiation, was also analyzed with the use of human STS cells. The mechanisms underlying the effects of the combination therapy were further analyzed in terms of apoptosis and the expressions of BCL2 family proteins such as MCL1 and BCL-xL. We used a subcutaneous xenograft tumor model to evaluate the therapeutic potential of combination OBP-702 and radiotherapy treatment.

Materials and Methods

Cell lines. Three human STS cell lines were used in this study: SYO-1 (synovial sarcoma), NMS-2 (malignant peripheral nerve sheath tumor), and HT1080 (fibrosarcoma) cells. The SYO-1 cells were previously established in our laboratory [15]. The NMS-2 cells [16] were kindly provided by Dr. Hiroyuki Kawashima (Niigata University, Niigata, Japan). The HT1080 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The SYO-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM). The NMS-2 cells were maintained in RPMI-1640 medium, and the HT1080 cells were maintained in Eagle's minimum essential medium. All media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were routinely maintained at 37°C in a humidified atmosphere with 5% CO_2.

Recombinant adenoviruses. The recombinant telomerase-specific replication-competent adenovirus OBP-301 (also known as suratadenoturev), in which the promoter element of the human telomerase reverse transcriptase gene drives the expression of the *E1A* and *E1B* genes, was previously constructed and characterized [17,18]. For the OBP-301-mediated induction of exogenous p53 gene expression, we generated OBP-702, in which a human wild-type p53 gene expression cassette was inserted into the *E3* region of OBP-301 [10,19]. The recombinant adenoviruses were purified using cesium chloride step gradients, and their titers were determined by a plaque-forming assay using 293 cells; viruses were stored at −80°C.

Cell viability assay. Cells were seeded on 96-well plates at a density of 1×10^3 cells/well at 24 h before irradiation or OBP-702 infection. In the monotherapy experiment, cells were irradiated at dosages of 0, 1, 5, or 10 Gy using an MBR-1520R device (Hitachi Medical Co., Tokyo) or infected with OBP-702 at a multiplicity of infection (MOI) of 0, 1, 10, or 50 plaque-forming

units (PFU)/cell. Cell viability was determined on day 4 after irradiation or day 5 after virus infection.

For the combination therapy, cells were infected with OBP-702 at an MOI of 0, 1, 10, or 50 PFU/cell. Twenty-four h after infection, the cells were irradiated at dosages of 0, 1, 5, or 10 Gy. Cell viability was determined on day 4 after irradiation using a Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's protocol. The effects of the combination of OBP-702 and ionizing radiation were analyzed by calculating the combination index (CI) using CalcuSyn software (BioSoft, Inc., Cambridge, UK).

Western blot analysis. Cells were seeded in 100-mm dishes at a density of 2×10^5 cells/well at 24 h before treatment. In the monotherapy experiment, cells were irradiated at the indicated dosages or infected with OBP-702 at the indicated MOIs. Cell lysates were obtained on days 1 and 4 after irradiation or 72 h after infection. In the combination therapy, cells were infected with OBP-702 for 24 h and/or irradiated at the indicated dosages. Cells were transfected with 10 nM MCL1 small interfering RNA (siRNA), BCL-xL siRNA, or control siRNA (Applied Biosystems, Foster City, CA, USA) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) for 48 h and/or irradiated at the indicated dosages. At 24 or 48 h after irradiation, whole-cell lysates that contained cytosol and nuclear proteins were prepared in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail (Complete Mini; Roche Applied Science, Mannheim, Germany).

Proteins were electrophoresed on 8-12% sodium dodecyl sulfate (SDS) polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes (Hybond-P; GE Health Care, Buckinghamshire, UK). Blots were blocked with Blocking-One (Nacalai Tesque, Kyoto, Japan) at room temperature for 30 min. The primary antibodies used were: rabbit anti-poly (ADP-ribose) polymerase (PARP) polyclonal antibody (pAb) (Cat. #9542; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-MCL1 monoclonal antibody (mAb) (#39224; Cell Signaling Technology), rabbit anti-BCL-xL mAb (#2764; Cell Signaling Technology), mouse anti-Ad5 E1A mAb (#554155; BD Bioscience, Franklin Lakes, NJ, USA), mouse anti-p53 mAb (#18032; Cell Signaling Technology), rabbit anti-phospho-p53 (Ser15) pAb (#9284; Cell Signaling Technology), and mouse anti–β-actin mAb (#A5441; Sigma-Aldrich, St. Louis, MO, USA). The secondary antibodies used were horseradish peroxidase-conjugated antibodies against rabbit IgG (GE Healthcare) or mouse IgG (GE Healthcare). Immunoreactive bands on the blots were visualized using enhanced chemiluminescence substrates (ECL Prime; GE Healthcare).

In vivo subcutaneous HT1080 xenograft tumor model. The animal experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine (no. OKU-2018319). All methods reported were applied in accord with the relevant guidelines and regulations, including the ARRIVE guidelines [20]. The experiments were conducted in accord with the Policy on the Care and Use of the Laboratory Animals, Okayama University. Under general anesthesia with 2% isoflurane, HT1080 cells (2×10^6 cells/mouse) were subcutaneously inoculated into the flanks of 5- to 6-week-old female BALB/C-*nu*/*nu* mice (CLEA Japan, Tokyo). When the tumors reached 5-8 mm in a diameter, the mice were treated with an intratumoral injection of OBP-702 (1×10^8 PFU/tumor) or phosphate-buffered saline (PBS) and/or irradiated at a dose of 2 Gy/tumor every week for three cycles starting at day 0 (n = 8 in each group). When irradiated, the mouse was placed prone in a custom-made holder that contained lead collimators to shield the upper half of the mouse. The perpendicular diameter of each tumor was measured twice each week, and the tumor volume was calculated using the following formula: tumor volume (mm^3) $= a\times b^2\times0.5$, where a is the longest diameter, b is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid. The body weight of each mouse was measured every week.

Histological analysis. The tumors excised from the mice anesthetized with 2% isoflurane and sacrificed were fixed in 10% neutralized formalin and embedded in paraffin blocks. Sections were stained with hematoxylin/eosin. Sections were also prepared for immunohistochemical examination with the use of a rabbit anti-Ki67 mAb (Abcam, Cambridge, UK). Immunoreactive signals were visualized using 3,3'-diaminobenzidine solution (Nichirei Bioscience, Tokyo), and nuclei were counterstained with hematoxylin. All sections were analyzed under light microscopy.

Statistical analyses. Data are expressed as the mean ± standard deviation (SD). The significance of

differences between two groups was assessed using Student's t-test. We performed a one-way analysis of variance (ANOVA) followed by Tukey's multiple-group comparison test was used to compare differences between groups in the animal experiment. Statistical significance was defined as a probability (p)-value < 0.05.

Results

In vitro *radiosensitizing effect of OBP-702 in human STS cells.* To evaluate the radiosensitizing effect of OBP-702 in STS cells, we analyzed the sensitivity to ionizing radiation in three human STS cell lines (SYO-1, NMS-2, HT1080). The viability of each cell line was measured by an XTT colorimetric assay on day 4 after the application of ionizing radiation. Compared to non-irradiated SYO-1 cells, the viability of the SYO-1 cells irradiated at 1Gy was < 50% (Fig. 1A). Conversely, the viability of HT1080 and NMS-2 cells was > 50% of that of the respective non-irradiated cells, even when the cells were irradiated at 10 Gy (Fig. 1A). Based on these results, we classified SYO-1 STS cells as relatively radiosensitive and HT1080 and NMS-2 cells as relatively radioresistant.

For the analysis of the sensitivity of STS cells to OBP-702, we measured the viability of each cell type on day 5 after infection by conducting an XTT assay. The results demonstrated that OBP-702 suppressed the viability of STS cells in a dose-dependent manner (Fig. 1B). The viability of all STS cells was < 50% of that of non-treated cells when infected at an MOI of 50 (Fig. 1B), indicating that all three STS cell lines were highly sensitive to OBP-702.

To further evaluate the combined effect of OBP-702 + ionizing radiation in human STS cells, we irradiated cells at 24 h after they were subjected to OBP-702 infection, and the cells' viability was assessed on day 4 after irradiation by an XTT assay. The combination of OBP-702 + ionizing radiation decreased the viability of human STS cells more efficiently than either single treatment (Fig. 1C). The calculation of the CI also revealed a synergistic antitumor effect of the combination therapy in all three STS cell lines (Fig. 1D). These results suggest that OBP-702 has the potential to enhance the radiosensitivity of STS cells.

The enhancement of ionizing radiation-induced apoptosis in human STS cells infected with OBP-702. To assess the mechanism underlying the OBP-702-mediated enhancement of cells' radiosensitivity, we next investigated whether OBP-702 would enhance the ionizing radiation-mediated apoptosis induction in STS cells. When SYO-1 cells were treated with ionizing radiation, the expression level of cleaved poly (ADP-ribose) polymerase (PARP) (C-PARP) increased in a dose-dependent manner on day 1 after irradiation. HT1080 cells showed an increased expression of C-PARP on day 4 after irradiation, but not on day 1 (Fig. 2A). When SYO-1 and HT1080 cells were treated with OBP-702, the expression level of C-PARP was similarly increased on day 3 after infection with OBP-702 (MOI 50) (Fig. 2B). When applied as part of combination therapy, OBP-702 increased the expression of C-PARP after ionizing radiation (Fig. 2C). These results suggest that OBP-702 enhances ionizing radiation-induced apoptosis in STS cells.

The suppression of BCL-xL is important for enhancing ionizing radiation-induced apoptosis in human STS cells. The suppression of anti-apoptotic BCL2 family proteins, including MCL1 and BCL-xL, is associated with the enhancement of ionizing radiation-induced apoptosis in cancer cells [4, 5]. Our group previously demonstrated that OBP-301 suppresses the expressions of MCL1 and BCL-xL, but not that of BCL2, in human osteosarcoma cells [11, 12]. Therefore, to evaluate the relationship between BCL2 family proteins and OBP-702-enhanced radiosensitivity in human STS cells, we analyzed the expression levels of MCL1 and BCL-xL in STS cells. We observed that the SYO-1 cells (with high radiosensitivity) showed low expressions of MCL1 and BCL-xL, whereas NMS-2 cells (with low radiosensitivity) showed a high expression of MCL1 and a low expression of BCL-xL (Fig. 3A). HT1080 cells (with low radiosensitivity) exhibited high expressions of MCL1 and BCL-xL (Fig. 3A). These results suggest that the expressions of MCL1 and BCL-xL proteins are associated with the radiosensitivity of STS cells.

To evaluate the roles of MCL1 and BCL-xL in the induction of apoptosis by combination therapy, we analyzed the expression levels of MCL1 and BCL-xL in HT1080 cells infected with OBP-702. The infection with OBP-702 at the highest dose (MOI 50) decreased the expressions of MCL1 and BCL-xL in HT1080 cells (Fig. 3B). Ionizing radiation decreased the MCL1 expression and increased the BCL-xL expression (Fig. 3C). OBP-702 efficiently decreased the expression

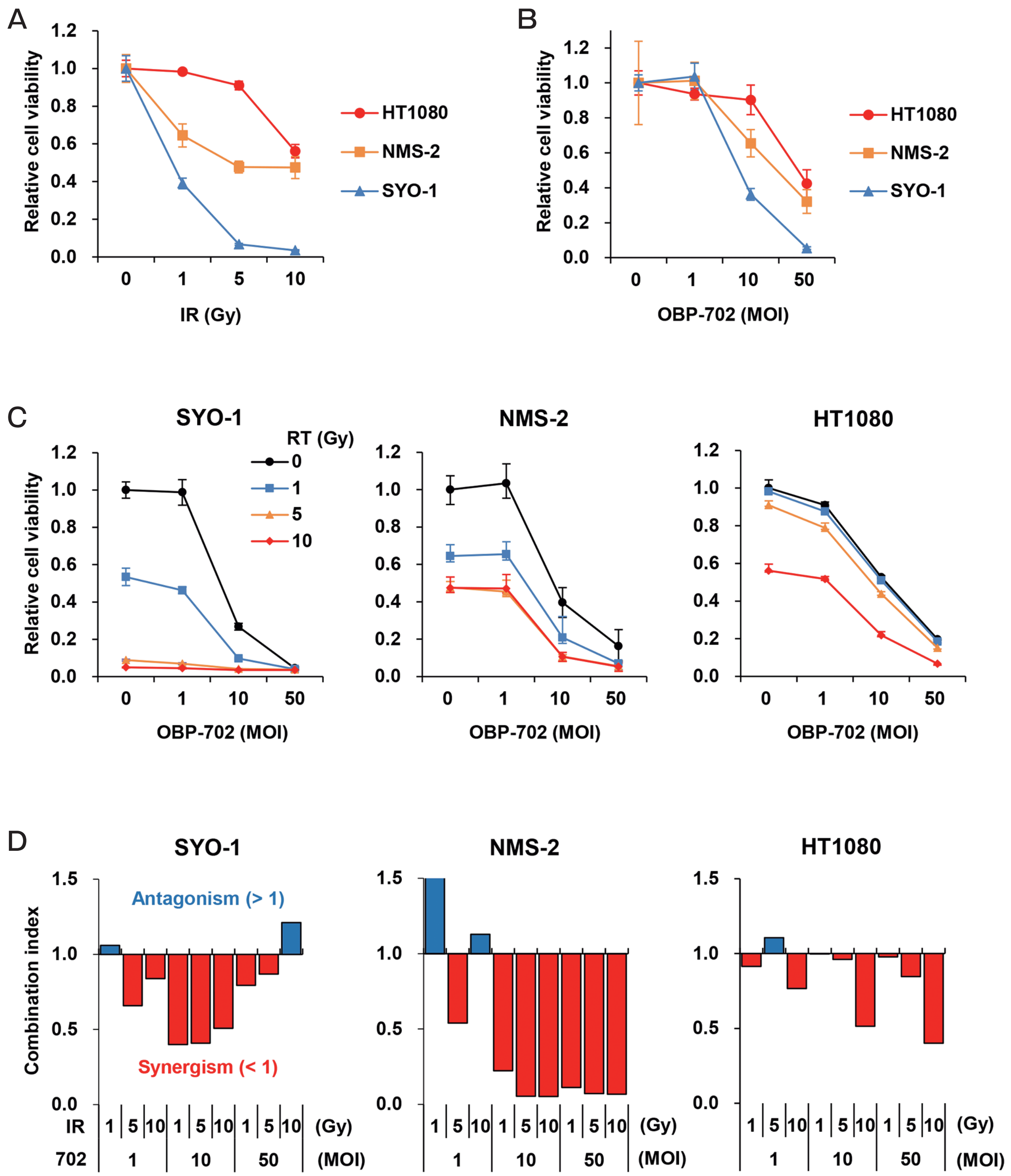

Fig. 1 OBP-702 promoted the antitumor effect of ionizing radiation in human soft-tissues sarcoma (STS) cells. A, The differing sensitivity to ionizing radiation in human STS cells. Three human STS cell lines (HT1080, NMS-2, and SYO-1) were treated with ionizing radiation (IR) at the indicated dose, and cell viability was assessed by an XTT assay on day 4 after the IR treatment. The data are mean ± SD (n=5); B, The differing sensitivity to OBP-702 in human STS cells. The human STS cell lines were treated with OBP-702 at the indicated dose, and cell viability was assessed by an XTT assay on day 5 after infection with OBP-702. Data are mean ± SD (n=5); C, The three STS cell lines were irradiated at the indicated dose 24 h after infection with OBP-702 at the indicated MOI, and cell viability was assessed by an XTT assay 4 days after the irradiation. Data are mean ± SD (n=5); D, The combination index (CI) was calculated using CalcuSyn software. Synergism and antagonism were defined as interaction indices of <1 and >1, respectively.

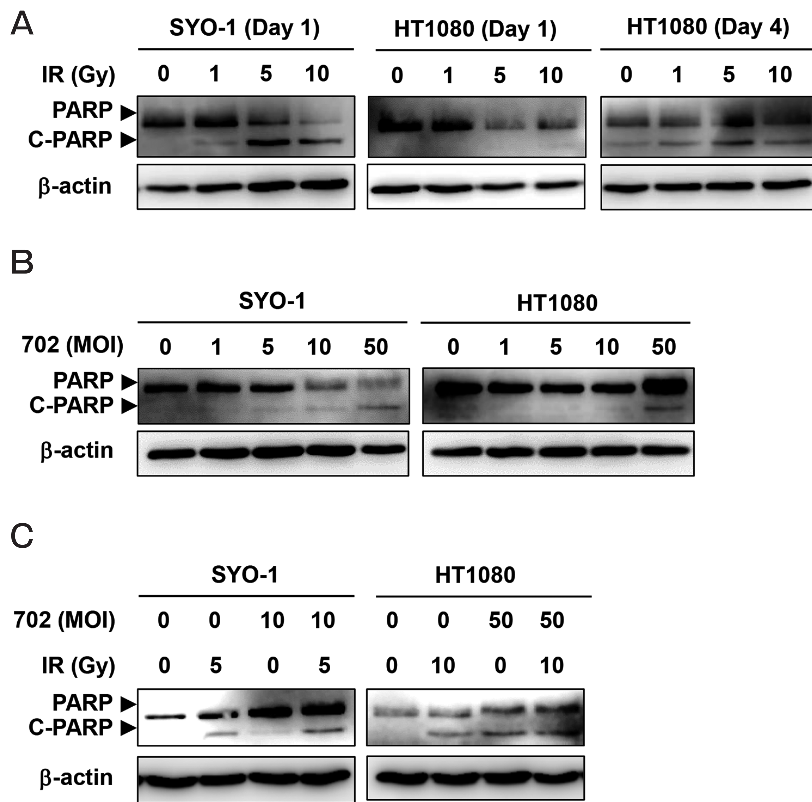

Fig. 2 OBP-702 promoted ionizing radiation-induced apoptosis in human soft-tissue sarcoma cells. **A**, SYO-1 and HT1080 cells were irradiated (IR) at the indicated dosages, and cell lysates were prepared on day 1 and day 4 after irradiation. Cell lysates were subjected to western blotting for PARP and cleaved PARP (C-PARP); **B**, SYO-1 and HT1080 cells were infected with OBP-702 at the indicated MOI for 72 h, and cell lysates were prepared and subjected to western blotting for PARP and C-PARP; **C**, SYO-1 and HT1080 cells were treated with OBP-702 and then irradiated at the indicated dosages 24 h after infection, and cell lysates were prepared on day 3 after infection and subjected to western blotting for PARP and C-PARP. β-Actin was assayed as a loading control.

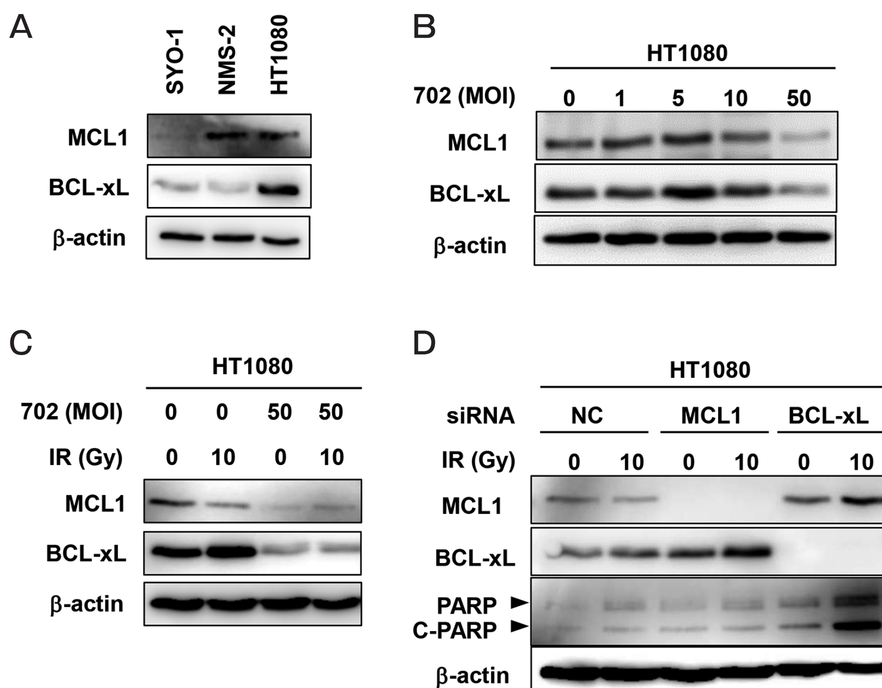

Fig. 3 BCL-xL suppression is involved in the OBP-702-mediated enhancement of apoptosis induced by ionizing radiation. **A**, Cell lysates were subjected to western blotting for MCL1 and BCL-xL; **B**, HT1080 cells were infected with OBP-702 at the indicated MOI for 72 h, and cell lysates were prepared and subjected to western blotting for MCL1 and BCL-xL; **C**, HT1080 cells were treated with OBP-702 and then irradiated (IR) at the indicated dosages 24 h after infection, and cell lysates were prepared on day 3 after infection and subjected to western blotting for MCL1 and BCL-xL; **D**, HT1080 cells were transfected with 10 nM MCL1 siRNA, BCL-xL siRNA, or control siRNA for 48 h and subsequently irradiated at the indicated dosages, and cell lysates were prepared and subjected to western blotting for MCL1, BCL-xL, PARP, and C-PARP. β-Actin was assayed as a loading control.

of both MCL1 and BCL-xL even when the cells were also treated with ionizing radiation (Fig. 3C). These results suggest that ionizing radiation increases BCL-xL expression, which is suppressed by OBP-702.

For a further evaluation of the roles of MCL1 and BCL-xL in ionizing radiation-induced apoptosis in HT1080 cells, the expressions of MCL1 and BCL-xL were suppressed by RNA interference in cells treated with or without ionizing radiation. In the ionizing radiation-treated HT1080 cells, MCL1 siRNA suppressed the MCL1 expression but increased the BCL-xL expression, and apoptosis was not enhanced (Fig. 3D). By contrast, BCL-xL siRNA suppressed the expression of BCL-xL and enhanced the ionizing radiation-induced apoptosis together with upregulations of cleaved and non-cleaved PARP in HT1080 cells, even though the MCL1 expression was conversely increased by ionizing radiation (Fig. 3D). These results suggest that the suppression of BCL-xL plays an important role in enhancing the induction of ionizing radiation-mediated apoptosis in STS cells.

OBP-702 is superior to OBP-301 and Ad-p53 in the suppression of MCL1 and BCL-xL expression. To investigate whether the activation of adenoviral E1A and p53 is involved in the suppression of MCL1 and BCL-xL expression, we evaluated the effect of recombinant adenoviruses in HT1080 cells. Non-armed OBP-301 was used to induce adenoviral E1A expression, and E1-deleted Ad-p53 was used to induce p53 expression. Since we had observed that Ad-p53 infection (MOI 500) induced the expressions of p53 and phosphorylated p53 (P-p53) in monotherapy (Fig. 4A), we treated HT1080 cells with Ad-p53 (MOI 1000) and/or OBP-301 (MOI 100).

The results showed that OBP-301 suppressed the expressions of MCL1 and BCL-xL, whereas Ad-p53 suppressed the expression of MCL1 and increased the expression of BCL-xL (Fig. 4B). Although combination therapy (*i.e.*, Ad-p53 + OBP-301) induced higher expressions of p53 and E1A than monotherapy, the expressions of MCL1 and BCL-xL were not suppressed (Fig. 4B). By contrast, OBP-702 infection (MOI 50) efficiently suppressed the expression of both MCL1 and BCL-xL and induced apoptosis in a manner consistent with the strong upregulations of E1A, p53, and P-p53 expression (Fig. 4B, C). These results suggest that OBP-702 has the potential to suppress the expressions of MCL1 and BCL-xL in STS cells more strongly than OBP-301 and Ad-p53.

Suppression of HT1080 tumor growth by combined treatment with ionizing radiation and OBP-702. Finally, to investigate the *in vivo* antitumor effect of combination therapy with ionizing radiation and OBP-702, we used a subcutaneous HT1080 xenograft tumor

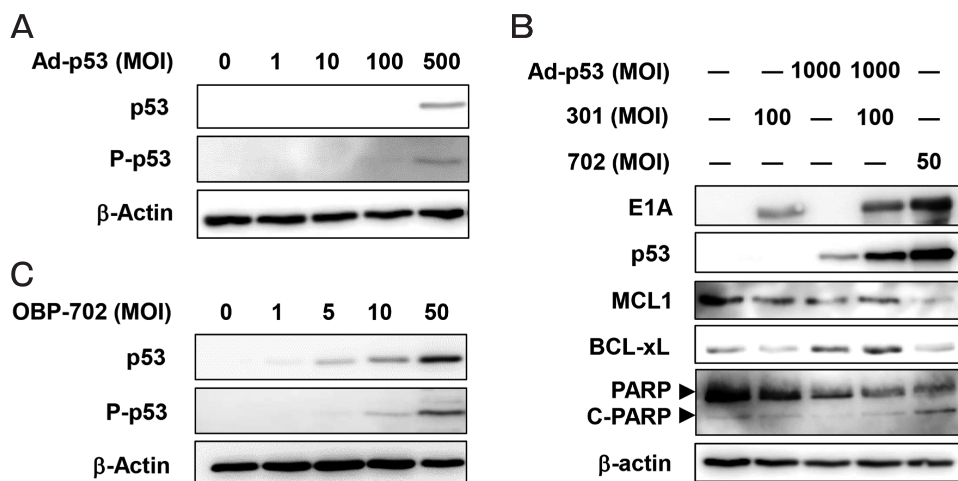

Fig. 4 OBP-702 suppressed BCL-xL expression more strongly than Ad-p53 and/or OBP-301. **A**, HT1080 cells were treated with Ad-p53 at the indicated dose for 72 h. Cell lysates were subjected to western blotting for p53 and phosphorylated p53 (P-p53); **B**, HT1080 cells were infected with Ad-p53 and/or OBP-301 or OBP-702 for 72 h in monotherapy and combination therapy. Cell lysates were prepared and subjected to western blotting for E1A, p53, MCL1, BCL-xL, PARP, and C-PARP; **C**, HT1080 cells were treated with OBP-702 at the indicated dose for 72 h. Cell lysates were subjected to western blotting for p53 and P-p53. β-Actin was assayed as a loading control.

model because HT1080 cells showed low sensitivity to ionizing radiation. Tumors were injected with OBP-702 or PBS (mock) and subsequently irradiated at 2 Gy every week for three cycles, and tumor growth was observed until day 28 after the first treatment (Fig. 5A).

The results revealed that the combination therapy of OBP-702 + ionizing radiation significantly suppressed tumor growth compared to the mock treatment and the monotherapy group (Fig. 5B). The mean body weight of the mock-treated mice was significantly decreased compared to those produced by the monotherapy or combination therapy (Fig. 5C). The histopathological analysis revealed that the combination therapy of OBP-702 + ionizing radiation resulted in a significant decrease in the number of Ki67-positive cells within the tumor tissues compared to the mock and monotherapy groups (Fig. 5D, E). These results suggest that OBP-702 has the potential to enhance the antitumor effect of ionizing radiation in STS cells.

Discussion

The standard treatment of STS patients includes surgery combined with pre- and/or post-operative radiotherapy, and the resistance to radiotherapy is a predictive factor in association with local and systemic recurrence, leading to the poor prognosis of STS patients [3]. Radiotherapy may offer the best chance for local tumor control for unresectable STSs, and thus enhancing the effectiveness of radiosensitivity is important for improving the clinical outcomes of STS patients. In this study, we investigated the radiosensitizing effect of OBP-702 in human STS cells, and we observed that OBP-702 efficiently suppressed the viability of STS cells in monotherapy or combination therapy together with ionizing radiation by suppressing the expressions of MCL1 and BCL-xL and promoting ionizing radiation-induced apoptosis.

In addition, treatment with BCL-xL siRNA, but not MCL1 siRNA, promoted the induction of ionizing radiation-mediated apoptosis in human STS cells. The combination therapy with OBP-702 + ionizing radiation significantly suppressed the growth of human STS cells compared to monotherapy. Together our findings suggest that OBP-702 is a promising antitumor reagent that could be used to enhance the radiosensitivity of human STS cells via the suppression of anti-apoptotic BCL-xL expression.

The combination treatment with OBP-702 promoted the radiosensitivity of human STS cells via the promotion of ionizing radiation-induced apoptosis (Figs. 1, 2). The underlying mechanism of the enhancement of ionizing radiation-induced apoptosis by OBP-702 involved the suppression of the expression of both MCL1 and BCL-xL (Fig. 3). Moreover, the suppression of BCL-xL by BCL-xL siRNA promoted ionizing radiation-induced apoptosis, whereas the MCL1 siRNA did not have this effect (Fig. 3). These findings suggest that BCL-xL plays a crucial role in the radiosensitivity of STS cells. Consistent with these findings, Sobol *et al.* demonstrated that among the BCL2 family proteins, BCL-xL plays a key role in regulating the radiosensitivity of human malignant tumor cells, including STS cells [21]. Moreover, de Graaff *et al.* showed that a pharmacological inhibition of BCL2 family proteins, including BCL-xL, enhanced the sensitivity of human STSs to chemotherapy [22].

Our investigation of the mechanism underlying the suppression of BCL-xL revealed that OBP-301, but not Ad-p53, suppressed BCL-xL expression in a manner similar to that exerted by OBP-702 (Fig. 4), suggesting the involvement of adenoviral E1A protein. Our research collaborators recently demonstrated that OBP-301 suppressed BCL-xL expression in radioresistant human oral squamous cell carcinoma cells by inhibiting the signal transducers and activators of transcription 3 (STAT3) signaling pathway [23]. As STAT3 reportedly promotes radioresistance in a variety of solid tumor cells [24], we speculate that OBP-702 may enhance radiosensitivity in human STS cells by suppressing the STAT3-BCL-xL pathway.

BCL-xL siRNA, but not MCL1 siRNA, enhanced the ionizing radiation-induced apoptosis and produced upregulations of both cleaved and non-cleaved PARP in HT1080 cells (Fig. 3D), suggesting that BCL-xL suppression enhances the ionizing radiation-induced DNA damage, resulting in the activation of PARP for DNA damage repair. Increasing evidence indicates that PARP inhibitors promote radiosensitivity in STS cells via an induction of synthetic lethality [25]. Although the mechanisms that underlie the ionizing radiation-induced PARP activation in the BCL-xL siRNA-treated STS cells remain unclear, our present findings suggest that a dual suppression of BCL-xL and PARP may be a promising antitumor strategy for the promotion of ionizing radiation-mediated synthetic lethality in STS cells.

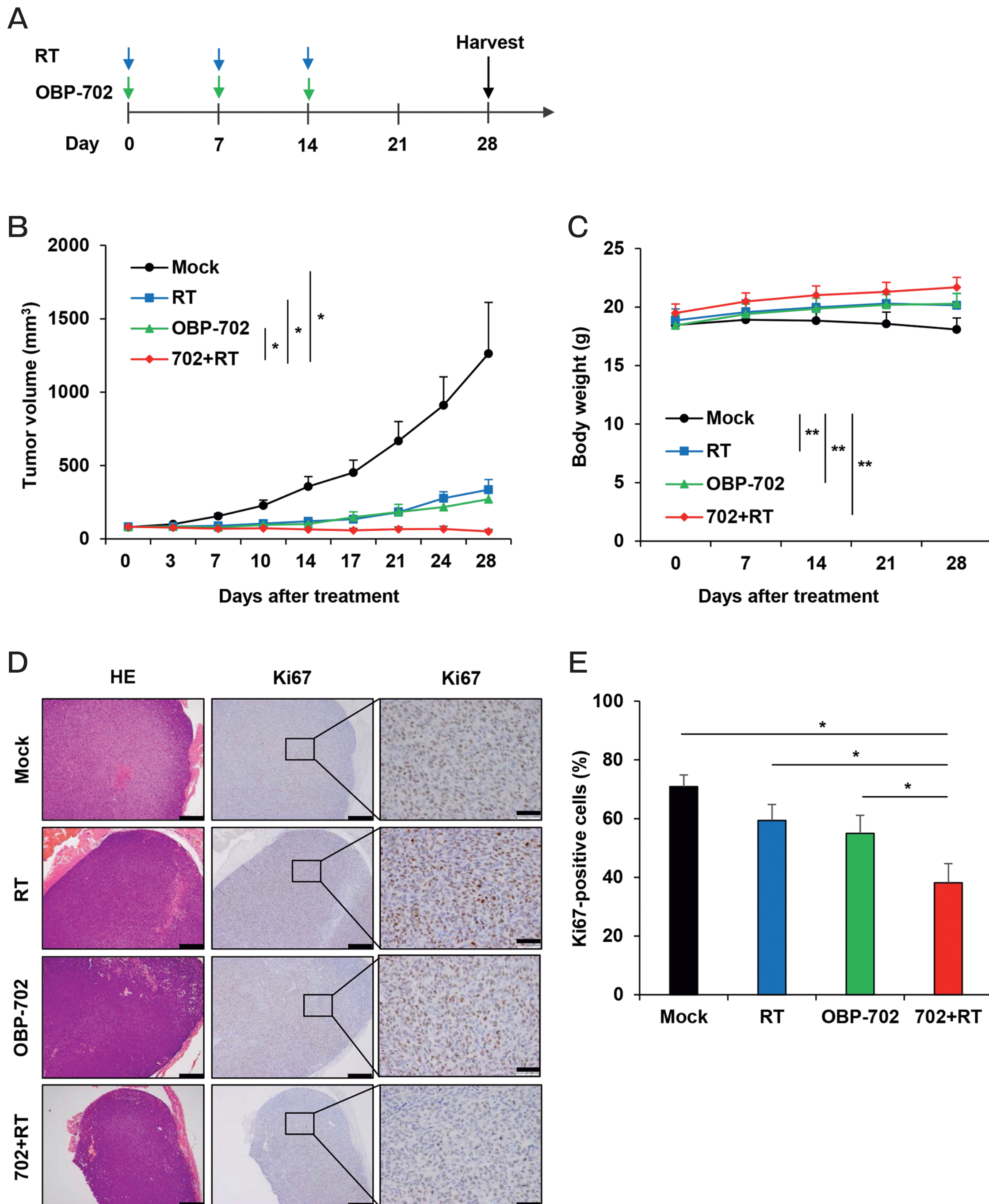

Fig. 5 OBP-702 promoted the antitumor effect of ionizing radiation in HT1080 xenograft tumor models. A,B, HT1080 cells (2×10^6 cells/mouse) were subcutaneously inoculated into the right flanks of mice. Tumor-bearing mice were treated with radiotherapy (RT) (2 Gy) after an intratumoral injection of OBP-702 (1×10^8 PFU/tumor) every week for three cycles (*arrows*: each treatment); C, The chronological changes in the body weights of HT1080 tumor-bearing mice treated with OBP-702 and/or radiotherapy; D, Histologic analysis of the HT1080 tumors. Tumor tissues were obtained on day 28 after the first treatment. Paraffin-embedded sections of HT1080 tumors were stained with hematoxylin and eosin (HE) solution or anti-Ki67 antibody. *Left and middle images*: low magnification of the HE and Ki67 staining. *Right images*: high magnification of the area outlined by the *black square* in Ki67 staining. Left and middle scale bars: 500 μm. Right scale bars: 50 μm; E, The number of Ki67-positive cells in tumor tissue was calculated using ImageJ software. Data are mean $\pm$ SD (n=8 in each group; *, $p<0.05$; **, $p<0.01$).

The present experiments demonstrated that OBP-702 monotherapy more effectively suppressed the expressions of MCL1 and BCL-xL compared to combination therapy with OBP-301 and Ad-p53, resulting in the difference in the apoptosis-inducing effect between the monotherapy and combination therapy. Regarding the mechanisms of these different effects, one possibility is that OBP-301 and Ad-p53 competitively infect to target cells by binding to the same adenovirus receptors. A virus-mediated upregulation of E1A and p53 in combination therapy may be not enough to induce apoptosis.

Another possibility is that the OBP-301-mediated BCL-xL downregulation was suppressed in the Ad-p53-treated cells. Tang *et al*. showed that Ad-p53 induced the expression of anti-apoptotic p21 in HT1080 cells [26], and Wu *et al*. demonstrated that p21 overexpression delayed the downregulation of BCL-xL in the oxidative stress-induced cells [27]. Since we observed that OBP-702 decreases the expression of p21 in human osteosarcoma cells [10], we speculate that in the present study, Ad-p53-mediated p21 upregulation may have suppressed the downregulation of BCL-xL expression in the OBP-301-treated cells.

Oncolytic adenoviruses are thought to promote the radiosensitivity of cancer cells by inhibiting the radiation-induced DNA damage response and DNA repair pathways [28]. In the immune-deficient nude mouse model examined in the present study, the intratumoral injection of OBP-702 significantly promoted the antitumor efficacy of radiotherapy against human STS cells (Fig. 5). However, oncolytic virotherapy and radiotherapy were recently shown to efficiently induce immunogenic cell death (ICD) and promote an antitumor immune response [29, 30], suggesting that antitumor immunity plays a critical role in the therapeutic potential of oncolytic virotherapy and radiotherapy. We recently demonstrated that p53-armed OBP-702 induces ICD against human and murine pancreatic cancer cells more strongly than non-armed OBP-301 or Ad-p53 [31]. Further experiments are thus warranted to evaluate the therapeutic potential of combination therapy with OBP-702 + radiotherapy against murine STS cells in a syngeneic mouse model.

In conclusion, the results of the present *in vitro* and *in vivo* experiments demonstrated that the p53-expressing oncolytic adenovirus OBP-702 suppresses the expressions of the anti-apoptotic MCL1 and BCL-xL proteins and enhances ionizing radiation-induced apoptosis in human STS cells. Oncolytic adenovirus–mediated p53 gene therapy may thus be a novel anticancer strategy that could be used to improve the antitumor effect of radiotherapy against human soft-tissue sarcomas.

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